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Human Subjects participated in these studies after giving free and informed voluntary consent. Investigators adhered to AR 70-25 and USAMRDC Reg 50-25 on the use of volunteers in research.

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ABSTRACT

An optimized additive solution was developed for the post-thaw preservation of red cells which contained adenine, glucose, disodium phosphate, and citrate buffer. This solution, called AS-17, was compared to Miles AS-3 solution in a clinical trial using 40 subjects (N=20 ea). Fresh-frozen red cells were thawed and deglycerolized after 1-18 months and subjected to a second period of storage in either AS-3 or AS-17 solution for as long as 3 weeks at refrigerator temperatures. Both solutions yielded red cells with 24-hour survivals in excess of 75%. The AS-17 solution produced improved pH, P50, and 2,3-DPG maintenance compared with the AS-3, but both solutions appear adequate for 3 weeks of post-thaw storage.

KEY WORDS:

blood storage, frozen blood, 2,3-DPG, ATP, post-thaw storage.

INTRODUCTION

The technology for preserving red cells in the frozen state has been available for two decades, but has been little used because it is expensive, and the thawed red cells must be used within 24 hours of thawing. Red cells may be cryopreserved in 6M glycerol for as long as 10 years at -80°C. After thawing, the cells must be deglycerolized by a procedure that includes washing with isotonic saline containing 200 mg/dl of glucose. Since this wash solution provides minimal nutritional support, and the washing procedure is not recognized as a "closed" procedure, the cells must be used within 24 hours or be discarded.

Post-thaw preservation has been studied to overcome the limited 24 hour post-thaw dating period. Moore et al. (1,2) showed that additives used for nonfrozen storage, i.e., ADSOL (Baxter Inc., Round Lake, IL), do not work well after cryopreservation, and that the nutrient mixtures required reformulation for optimal post-thaw preservation. Myhre and Marcus (3) evaluated in vitro several currently available red cell preservative solutions for their ability to sustain red cells in a post-thaw state and found CPDA-1 to be the most promising solution. Subsequently, they showed that CPDA-1 supplementation yielded acceptable red cell survivals after 3 weeks of 4°C post-thaw storage (3).

In this report, we discuss development of an additive solution based on our earlier data (1), and show results of the clinical trial with this solution and the currently available

AS-3 solution of Miles, Inc. (Covina, CA).

MATERIALS AND METHODS

All units of blood were drawn from normal healthy adult volunteers after obtaining informed consent. The human trials protocol was approved and monitored by the Letterman Army Institute of Research Human Use Committee, the Army Medical Research & Development Command Human Use Committee, and the Miles Laboratory Clinical Studies Department. Blood was collected into CPDA-1 using oversized (800 ml) triple bag sets, and frozen within 2 hours by the standard military protocol (4,5). All units were thawed in a 40°C water bath and deglycerolized with a Haemonetics Model 115 cell washer (Braintree, MA), using the military protocol (5).

Test Solutions

Two test solutions were used in this study. One was the Miles, Inc. (Cutter) AS-3 Solution consisting of 1.10 g glucose, 0.042 g citric acid, 0.276 g monobasic sodium phosphate, 0.410 g sodium chloride, 0.030 g adenine, and 0.588 g sodium citrate in 100 ml of water @ pH 5.8. The second solution, denoted AS-17, consisted of two parts which were mixed just prior to use; the first part (17A) contained 0.46 g dibasic sodium phosphate, and 0.56 g sodium citrate in 10 ml of water. The second part (17B)

contained 0.030 g adenine, 1.20 g dextrose, and 0.90 mg of citric acid in 50 ml of water. The pH of the combined solutions was 8.3. Sterile bags of the AS-17 solution were prepared by Miles, Inc. for our use in this study.

In Vitro Studies

Each in vitro study was done by thawing 5 units of red cells, deglycerolizing the cells, and centrifuging (2200 rpm x 10 min) to repack the cells to a hematocrit of about 80%. Supernatant saline was expressed off. Then the test additive solution was added by sterile filtration through a 0.22μ filter, and mixed. All units were stored in 600 ml polyvinyl chloride transfer packs. A time zero sample was taken and the red cell units were stored at 4°C for 3 weeks with weekly mixing and sterile sampling. At the end of the studies all bags of blood were cultured to assure sterility. Culturing was done for 1 week in thioglycolate broth, at 4° and 37°C, both aerobically and anaerobically.

In Vivo Studies

Forty subjects participated in this study. A unit of red cells from each subject was stored frozen for 1 to 18 months, thawed, deglycerolized, repacked to Hct=80, then mixed by sterile

splicing with one of the two test solutions (20 subjects each). The sterile closed-bag systems were combined with a Haemonetics SCD 312 sterile splicer (Braintree, MA). A time zero sample was aseptically drawn for in vitro analysis, and the red cells were stored at 3°C for either 14 or 21 days, thus, N=10 for each time and for each solution. On the 14th or 21st day 20 ml of red cells were tagged with 20 µCi of chromate using the single label procedure of Moroff, et al. (6). The chromium was purchased in 20 µCi doses from Mallinckrodt Pharmaceutical Corp(San Francisco, CA). On the day of infusion, a second sample was also taken for supporting in vitro tests. Eight days prior to infusion 20 ml of the stored red cells were removed via a satellite bag and cultured by the hospital microbiology lab to assure sterility. Culturing was done as before. Survival data were analyzed as previously reported (7). Pregnancy tests were done on all females two days prior to infusion.

In Vitro Assays

We measured P50 on a Hemox-analyzer (TSC Medical Products, Huntington Valley, PA) and "blood" pH under anaerobic conditions on a Corning Model 170 (Medford, MA) pH/blood gas analyzer. Red cell morphology index was measured using the method of Hogman (8). Assays for red cell ATP, 2,3-DPG, glucose, and supernatant hemoglobin, K^+, and Na^+ were done as before (7). Statistical analyses (t-tests) were done either on EXCEL 4 (Microsoft,

Redmond, WA), or NCSS 5.0 (Kaysville, UT).

RESULTS

In Vitro Development of AS-17

Our earlier studies (1) showed that post-thawed red cells stored better in an additive solution which had different quantities of nutrients than are used in current additive solutions, and that by using the tribasic sodium phosphate to elevate pH, we could improve in vitro storage parameters. This system was optimized using experimental design techniques as described previously (9). The resulting solution produced nearly 100% retention of red cell ATP and 2,3-DPG for 3 weeks (data not shown). However, because of the high pH of the trisodium phosphate (additive pH = 11.3) the solution was not compatible with the PVC plastic bag systems used in blood processing. Many attempts were made to find an answer to this packaging problem, but none were deemed successful. This incompatibility precluded development of the trisodium phosphate solution (TSP) and forced us to substitute disodium for TSP to produce a stable solution (pH = 8.3). This modified solution was designated as AS-17 for the clinical trial.

The results of in vitro studies with AS-17 and AS-3 are shown in Figure 1. The disodium phosphate (DSP) containing additive solution was significantly superior to AS-3 in maintenance of both red cell ATP, which partially correlates with

viability, and red cell 2,3-DPG which correlates with red cell function. Cell lysis, morphology, and supernatant K were similar to each other and within the ranges accepted for nonfrozen red cell storage systems (data not shown).

In Vivo Study

The in vivo, or clinical trial, study was designed to answer two major questions. What are the time limits for using AS-3 as a post-thaw preservative? And, as the in vitro data suggest, is the AS-17 solution superior to AS-3, in both the length of storage time it provides and the functional properties of the resulting red cells? Storage time is determined by the FDA requirement that the mean 24-hour post-transfusion survival of the red cells be at least 75%.

Table 1 shows the results of the in vivo comparisons of AS-3 and AS-17. Both solutions exceed the FDA cutoff of 75% survival at 14 and 21 days. There are no significant differences in the two solutions at either time period; however, the AS-17 appears slightly better at 21 days, where one outlier (S = 50%) lowered the mean value and spread the variance. Life span of the infused cells is normal and not significantly different between the solutions. The Index of Therapeutic Effectiveness, defined as freeze-thaw recovery times 24-hour survival is also acceptable through 21 days and was not significantly different between the two solutions.

The in vitro data are presented in Table 2. Freeze-thaw

recovery was excellent, averaging 94% of the red cells. The pH of cells stored in AS-17 was significantly higher than cells stored in AS-3 due to the higher initial pH and buffering capacity of the disodium phosphate. Hematocrit was also higher in the AS-17 units due to the reduced volume of the additive solution (60 vs 100 ml). The AS-17 samples also showed significantly higher levels of 2,3-DPG at both times, but the ATP values were significantly improved only at day 14. Supernatant potassium levels were similar after correction for hematocrit differences and were similar to other non-frozen additive systems on a load-per-unit basis. No data was collected at the 7-Day storage time. All units were negative to bacterial culturing from samples drawn 8 days prior- and 1 day post-infusion.

DISCUSSION

The ultimate goal of our work is to produce a post-thaw preservation solution which will allow deglycerolized red cells to be stored a second time at refrigeration temperatures as long as 3 weeks. This extended time would greatly increase the utility of frozen cells. The US military has a program to stockpile frozen group O cells for emergency use. A 3 week dating on the thawed product would improve logistical flexibility for using these cells. Our earlier studies indicated that frozen-thawed cells do not store as well as fresh cells, and the currently used additive solutions performed poorly during in

vitro studies. We decided to reoptimize the commonly used mixtures of red cell nutrients to obtain a mixture which both maintained red cell ATP, enhanced the probability of good viability, and maintained 2,3-DPG to ensure good oxygen delivery. Such a system was developed, as mentioned above, partially by using TSP to provide phosphate and elevated pH. This solution had a pH = 11.3, which, when mixed with blood, produced a final pH of 7.4 at 37°C, and maintained at least 71% of the 2,3-DPG after 3 weeks. However, the plastics used in blood bags are not stable at pH 11.3, and after many attempts (at Miles, Inc.) we were unable to produce a workable combination of a stable solution container and a closed bag unit. We compromised by substituting DSP for TSP in the formula, thereby reducing the solution pH to 8.3, which was stable in a PVC blood bag. The "cost" was lower pH and loss of half our ability to maintain 2,3-DPG.

A second issue we resolved was red cell lysis. Fresh red cells are stored in preservation systems containing either mannitol or citrate to retard lysis. Both are effective. However, after red cells have been frozen and thawed, mannitol is much less effective in retarding lysis. Citrate must be used to control lysis during extended post-thaw storage.

Since the modifications in our additive solution were moving it closer to the formulation of AS-3 we did the in vitro comparison study of these two solutions. From this study we predicted that AS-17 would produce better viabilities after 21

days of storage, and that AS-17 would be significantly better than AS-3 at delivering oxygen throughout the 21-day storage period. From the in vivo study we found that both solutions produced good viability to 21 days, exceeding the FDA standard of a 75% mean. The superior ATP levels of AS-17 did not produce significantly better viabilities at 21 days. Although P50, pH, and 2,3-DPG were better maintained in AS-17, and even though the difference was statistically significant, it is probably not clinically significant. However, ongoing deglycerolization studies in our laboratory indicate that modifications in the wash solution may enhance the ability of AS-17 to maintain pH and 2,3-DPG. These studies will be reported later.

Our data indicate that either AS-3 or AS-17 may be used to preserve red cells for 21 days after thawing. These systems are superior to the CPDA-1 approach, which is limited to 2 weeks. Our results for AS-3 are superior to those reported by Valeri et al.(10) who found 14-day survivals of 76% in AS-3. However, Valeri had pre-stored the fresh cells up to 1 week at 4°C prior to freezing, which may have had a negative effect upon their final viability.

Another important factor in optimizing post-thaw cell quality is the need to repack the red cells to a Hct of 70-80% prior to adding any type of additive system. This step dramatically reduces lysis (as much as 20-fold), although citrate is still required. The reasons are not clear, but higher citrate concentrations used without repacking do not overcome the higher

lysis.

These studies demonstrate the potential of 3-week post-thaw preservation of deglycerolized red cells. A standardized, closed method must still be developed to repack the deglycerolized cells and combine them with the additive system because our approach of sterile splicing and manual centrifugation is too cumbersome for routine high-volume use. The ideal approach would be to modify the deglycerolizing procedure to produce a packed end product. Studies in this area are in progress in several labs. Routine post-thaw preservation would make the military frozen stockpile system very workable for unplanned emergencies, and should appeal to many civilian blood centers for similar reasons.

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Figure Captions

Figure 1. Mean levels of ATP and 2,3-DPG during an in vitro comparison of post-thaw preserved red cells stored in Miles AS-3 solution (Nutricel) or in an experimental solution (AS-17) containing phosphate as the disodium salt (DSP). N=5 each.

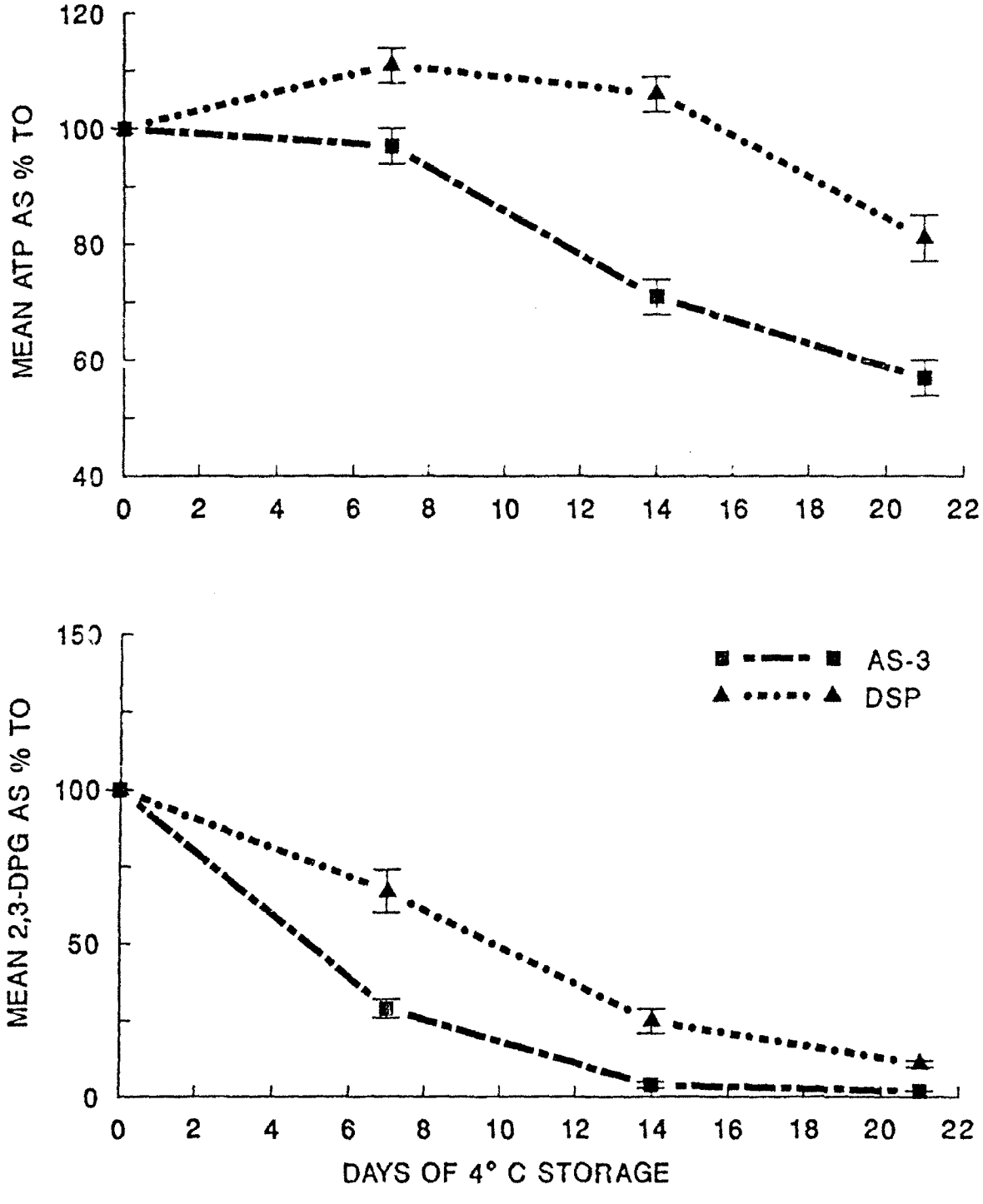
120
100
80
60
40
MEAN ATP AS % TO
0
2
4
6
8
10
12
14
16
18
20
22
150
100
50
0
MEAN 2,3-DPG AS % TO
AS-3
DSP
DAYS OF 4° C STORAGE

Table 1

MEAN IN VIVO DATA ±SD

SAMPLE	24-HR SURVIVAL (Percent)	LIFE-SPAN (Days)	ITE (Percent)
AS-3, 14 DAY	85±5	61±11	80±6
AS-3, 21 DAY	77±8	65±27	72±9
AS-17, 14 DAY	86±4	67±7	82±5
AS-17, 21 DAY	79±11	77±24	73±12

(N=10 each)

Table 2

Mean ± SEM In Vitro Data From Clinical Trials Samples

Assay Parameter	Post-Thaw AS-3	Post-Thaw AS-17	Day 14 AS-3	Day 14 AS-17	Day 21 AS-3	Day 21 AS-17
% P-T Recovery	94±1	94±1	--	--	--	--
pH @ 37°	6.71*	6.95	6.54	6.66	6.43	6.56
P50 mmHg	28±0.5	28±0.5	17±0.5	20±0.4	16±0.8	17±1.0
Morph Index %	100±0	100±0	86±1	86±2	82±2	79±2
Hct %	61±3	67±1	60±2	66±2	60±1	67±2
% RBC Lysis	0†	0	0.27	0.44	0.57	0.54
Glucose mg/dl	425±4	575±12	349±5	425±10	340±9	422±14
ATP µmol/g.HB	3.70±.1	3.84±.1	2.40±.2	3.51±.2	2.19±.1	2.89±.1
DPG µmol/g.HB	11.87	11.69	0.92	3.84	0.34	1.17
Super-K meq/L	3.7±.04	3.7±.04	43±3	52±3	46±1	60±1

* SEM of pH data all ≤0.01

† Time zero plasma HB (from wash solution) set at zero for calculation of post-thaw RBC lysis. All SEMs ≤0.10.

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